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Our aim is to identify molecules that mediate the specific homing of metastatic tumor cells to bone. Our approach involves the use of random peptide libraries expressed on the surface of filamentous phage as well as novel expression cloning strategies using immortalized bone marrow stromal and endothelial cells to detect the binding of COS-1 cells transfected with cDNAs from the bone metastatic MDA-MB-231 breast cancer cell line.

Using both these approaches we have successfully identified 10 peptides by *in vivo* phage display and two novel cDNAs by expression cloning and work continues on the characterization of these molecules. This past year we also developed a new *in vivo* targeting strategy based on injecting transfected COS-1 cells into mice and recovering the transfected plasmids from those cells that home to the bone marrow. With this new strategy we have isolate 15 new promising clones.

These experimental approaches will lead to the discovery of molecules involved in metastasis which remains today one of fundamental unresolved problems in tumor biology. Furthermore, identification of bone specific homing sequences could enable us to design vectors to be used in gene therapy of genetic diseases effecting bone and/or to block bone metastasis.

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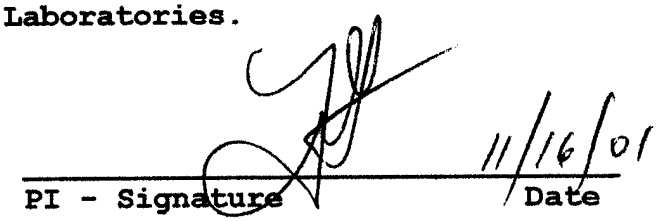

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5. INTRODUCTION

More than 70 % of breast cancer patients show bone metastasis at the time of autopsy. Bone metastasis is a devastating symptom, which can cause bone fractures, severe pain, hypercalcemia and/or neurological paralysis. Despite its clinical importance, the mechanisms that cause bone preferential metastasis are not well understood. Currently no efficient methods to prevent or treat bone metastasis are available. The establishment of metastasis requires multiple steps. First cancer cells within the epithelium of the primary organ must be released. After reaching the blood vessels, the cells rupture the basement membrane and are released into the circulation. Floating cancer cells initially adhere to endothelial cells of the secondary organ and then invade the stroma. If they successfully proliferate within the secondary organ, metastasis is established (Hynes and Lander, 1992). We have focused on the initial adhesion of breast cancer cells in the secondary organ, bone marrow. We hypothesize that breast cancer cells released into the circulation adhere to the stromal cells and/or endothelial cells in the bone marrow through specific binding(s). Although little information is currently available about the molecules involved in this step of breast cancer, in other cancers receptor/ligand-like specific interactions mediating metastasis have been characterized. For example, VLA-4 ($\alpha_4\beta_1$) integrin on skin melanoma binds to VCAM-1 (vascular cell adhesion molecules 1) expressed on activated endothelial cells, and Sialyl Lewis X on colon cancer recognizes E-selectin expressed on hepatic endothelium (Zetter, 1993). Prostate cancers also show a high incidence of bone metastasis. Haq et al. showed that a rat prostate cancer cell line contained a population preferentially bound to bone marrow-derived endothelial cells (Haq et al.,1992). Tumors of prostate cancer patients who developed bone metastasis and those of the patients who developed lymph node metastasis presented different lectin binding patterns indicating the existence of molecules that help to determine the preference for the secondary organ (McMahon et al.,1994). Generally, it is believed that floating cancer cells bind first to the endothelial cells of the secondary organ; however, we decided to focus on both the bone marrow stromal cells and endothelial cells for our studies. Bone marrow has a unique structure called the "bone marrow sinus", which has an intermittent basement membrane allowing matured hematopoietic cells to be released into circulation (Apaja-Sarkkinen et al.,1986). This observation likely indicates that cells in circulation can easily enter the bone marrow cavity. Therefore, endothelial cells may not be the only cells that floating breast cancer cells first interact with. Bone marrow stromal cells function in the maturation and sorting of hematopoietic cells. It has been suggested that VCAM-1 expressed in certain bone marrow stromal cells mediates adhesion to hematopoietic cells (Jacobson et al.,1996). Isoforms of CD44, which is one of the cell adhesion receptors expressed in bone marrow stromal cells, may be involved in metastasis (Lesley et al.,1993). VLA-4, a receptor of VCAM-1, expressed in myeloma cells binds to VCAM-1 on the bone marrow stromal cells suggesting that VLA-4 /VCAM-1 interaction facilitates homing of myeloma to bone (Michigami, et al.,2000).

We are using complementary approaches to try to uncover molecules that home specifically to bone. One is a novel approach developed at our institute that makes use of random peptide libraries expressed on the surface of filamentous phage in order to identify peptides that may confer preferential homing properties. A second approach is a more classical panning strategy, whereby a metastatic breast cancer cell line is used to generate a cDNA library that is then expressed in a mammalian expression system. To do this, we have generated a number of immortalized bone marrow stromal and endothelial cell lines and developed a novel binding assay to identify the binding of transfectants onto the bone marrow cell lines. Still a third approach has been developed this past year, which involves the in vivo targeting of transfected COS-1 cells followed by recovery of the plasmids form those

transfectants that home specifically to the bone marrow. All three of these experimental approaches may lead to uncovering the basic mechanisms of bone metastasis by cancer cells which remain today one of the fundamental unresolved problems in tumor biology. In a more general application, identification of bone-specific homing sequences would enable the design of vectors to be used in gene therapy of genetic diseases affecting bone.

6. BODY

The approved tasks for this project include:

1) To generate and screen random peptide libraries in vivo to identify sequence motifs homing specifically to bone.

2) To ascertain if the peptide sequences exist in the context of full length cDNA molecules present on metastatic breast cancer cells

3) To identify and clone the receptors on the surface of bone marrow endothelial cells that are recognized by the homing peptides.

During this third year of support we have continued to obtain information on the previously identified clones while we have also optimized an alternative *in vivo* targeting strategy and uncovered new candidate sequences. I will now summarize our major advances :

Results from expression cloning experiments using a bone marrow stromal cell line:

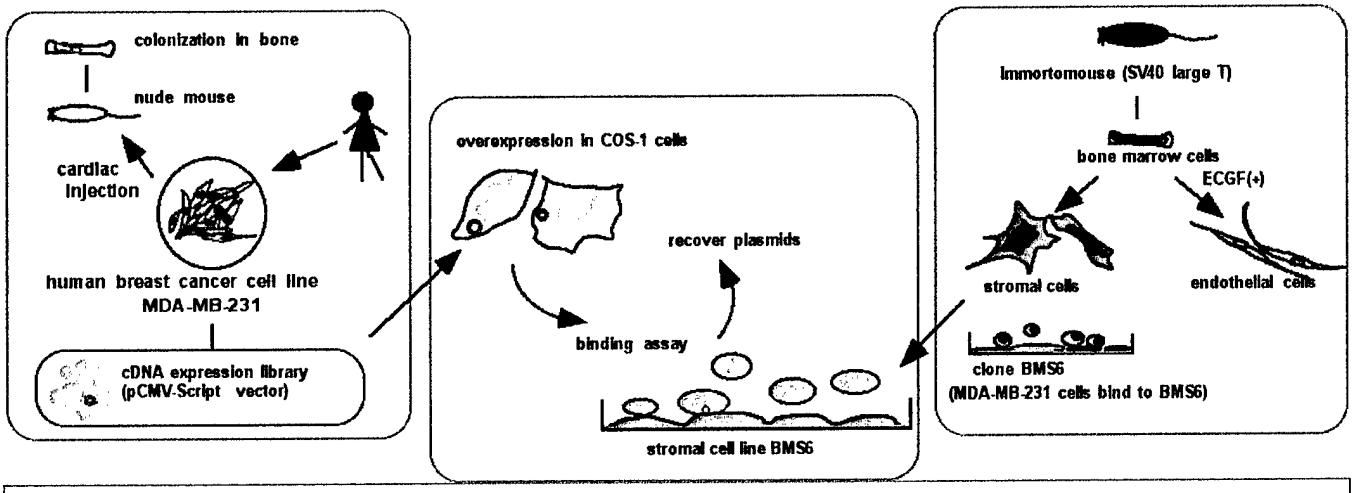

Fig. 1 Bone marrow cells were isolated from the Immortomouse and clone BMS6 and BME5 were chosen due to their ability to capture MDA-MB-231 cells. cDNA expression library of bone metastatic breast cancer MDA-MB-231 was expressed in COS-1 cells. Cytoplasmic DNA from the COS-1 cells bound to BMS6 was recovered and this cycle was repeated.

We developed an expression cloning strategy designed to identify molecules that bind to bone marrow cells (Fig. 1). The establishment of this cloning system required three steps. (I) First, we immortalized bone marrow stromal cells and endothelial cells. These cells are considered the target cells to which breast cancer cells bind. (II) Secondly, we constructed a cDNA expression library of the human breast cancer cell line MDA-MB-231 (231), (III) Finally, we expressed the library, incubated those cells with our immortalized bone marrow stromal cells, and recovered cDNA clones from the COS-1 cells that bound to the stromal cells.

Although the breast cancer cell line 231 was established from pleural fluid from a breast cancer patient (Cailleau et al., 1974), it is believed to maintain "bone metastatic characteristics", since it can colonize the bone marrow of nude mice if injected through the left cardiac ventricle, while the control breast cancer cell line MCF7 can not (Yoneda et al., 1994). We previously observed that 231 cells contained a subpopulation that bound to primary cultures of mouse bone marrow cells. Firstly, we established cell lines isolated from the bone marrow of the Immortomouse (Charles River Laboratories, Wilmington, MA), a transgenic mouse ubiquitously expressing SV40 large T antigen. Cells isolated from the Immortomouse proliferate under permissive conditions i.e., in the presence of 100 units/ml IFN-γ n the media at 33°C (Jat et al.,1991). After single-cell cloning of the primary culture, seven stromal cell clones and nine endothelial cell clones were obtained, and their morphology and the expression patterns of marker proteins were examined. Endothelial growth factor supplement (Sigma, St. Louis, MO) was used for the establishment of endothelial cell clones (Haq et al.,1992). We have chosen one stromal cell line (BMS6) and one endothelial cell line (BME5) because of their superior binding to 231 cells. BMS6 cells express stromal cell markers, such as alkaline phosphatase and CD44, and BME5 cells express endothelial cell markers, alkaline phosphatase, von Willebrand factor and VCAM-1. Fig. 2 shows alkaline phosphatase activity in BMS6 and BME5. Next, we constructed an expression library of the 231 cell line using an expression vector pCMV-Script (Stratagene, La Jolla, CA). This plasmid library has an average insert size of 1.3 kb and a size of is 4.0×10^5 primary colony forming units.

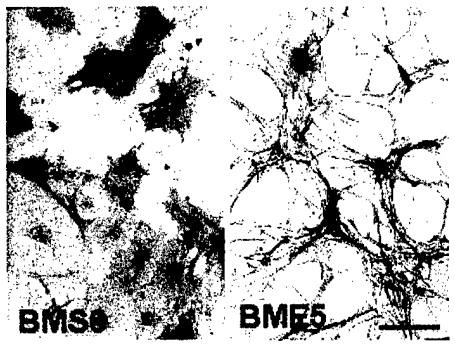

Fig. 2 BMS6 and BME5 stained for alkaline phosphatase (red). Nucleus were stained with methyl green.

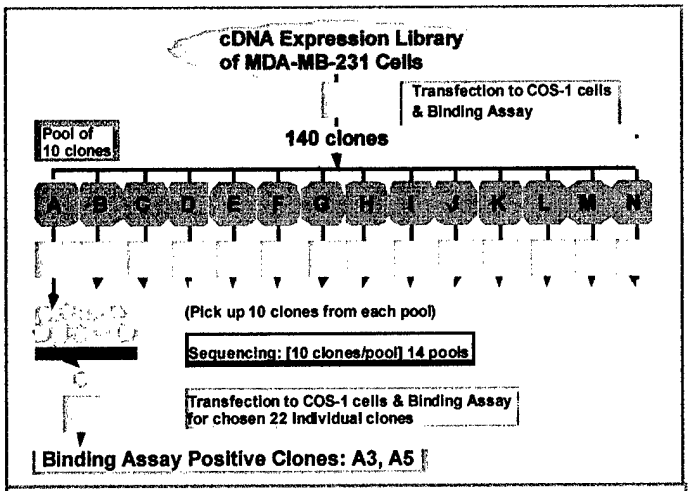

Fig. 3 Recovered 140 clones were divided into 10 groups. DNA of each group was transfected into COS-1 cells again, and 10 clones from each group were analyzed. Twenty-two clones were individually transfected, and binding was compared.

The process of the expression cloning is illustrated in Fig. 3. Forty μg of plasmid DNAs of the library were transfected into COS-1 cells by electroporation, and 60 hours later, the cells were harvested with 1% collagenase A (Roche, Indianapolis, IN) solution containing 0.005% trypsin inhibitor (Sigma, St. Louis, MO) and 5mM $CaCl_2$. BMS6 cells were fixed with ethanol and washed with serum free medium. The COS-1 cells suspended in culture medium containing 0.11 % EDTA were incubated on top of the BMS6 cells under mild shaking for 30 min. After eight times of washing with 0.02 % EDTA-PBS, amplified DNA was recovered from the cytoplasm of bound COS-1 cells and used for transformation of XL-10 Gold Ultracompetent Cells (Stratagene, La Jolla, CA).

A total of 140 clones were collected after the first cycle, cultured individually, and divided into ten groups (A to N). Fifty μg of DNA from each group were transfected into COS-1 cells again, and amplified plasmids from the bound COS-1 cells were recovered. Ten cDNA clones from each group

were sequenced, and the sequences were compared to the databases. Twenty-two clones were chosen because they appeared more than twice, had large insert (more than 1 kb), were unknown sequences, or were membrane proteins. Each cDNA clone was individually transfected into COS-1 cells, and the cells were processed as descried in the previous step. After eight times of washing with 0.02 % EDTA-PBS, the samples were stained with trypan blue. Live bound COS-1 cells appeared as round, shining cells, while the target fixed cells were stained with trypan blue as shown in Fig.4. Clones A3 and A5 showed significant binding, and clone H7 was considered as negative.

Fig. 4 Binding assay. COS-1 cells transfected with clone A3, A5, and H1 incubated with fixed BMS6 cells. MDA-MB-231 cells were positive control. Live bound cells appear in white round shape.

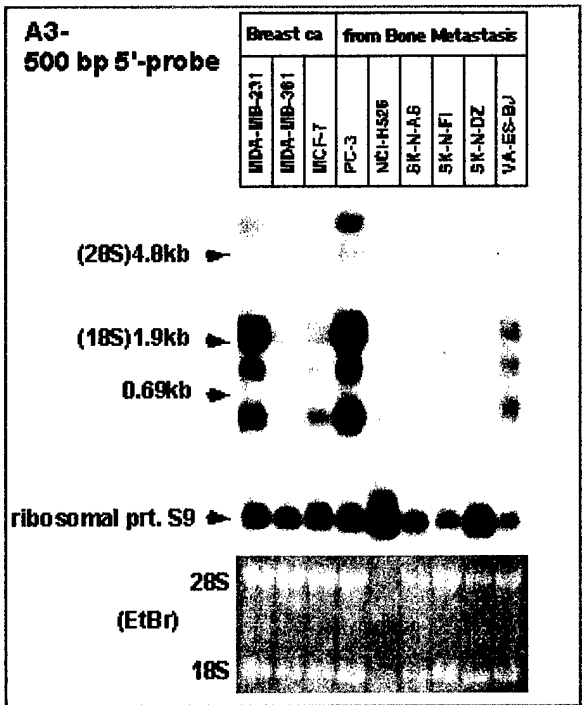

Fig. 5 Northern blot with 500 bp probe of A3. Ten μg of total RNA was loaded in each lane. MDA-MB-231, MDA-MB-361, MCF7, breast cancer; PC-3, prostate cancer; NCI-H526, lung cancer; SK-N-AS, SK-N-FI, SK-N-DZ, neuroblastoma; VA-ES-BJ, sarcoma.

Clone A3: Clone A3 is a 1.1 kb fragment containing entire coding sequence of CD59, which is known as a GPI anchored membrane protein. CD59 binds to complement 8 and 9. It is suggested that CD59 expressed on the surface of cancer cells protects them from complement attack (Chen et al., 2000). However, no function for CD59 has been proposed in cell-cell adhesion. Northern blot hybridization with a probe of clone A3 to human cancer cell lines is shown in Fig. 5. A3 is highly expressed in 231 and a prostate cancer cell line PC3 established from bone metastasis. The expression of A3 is lower in MDA-MB-361 cells, which are a breast cancer line isolated from brain metastasis, and breast cancer cell line MCF7, which does not colonize the bone marrow in nude mice (Yoneda et al.,1994). The remaining cell lines analyzed were non-breast cancers but isolated from bone metastasis. The multiple bands seen in each lane as shown in Fig. 5 are known to be due to alternative poly-A adenylations (Tone et al., 1992). This result suggests that A3 (CD59) can be a candidate molecule for mediating bone preferential homing of both breast cancer and prostate cancer.

Clone A5: The sequence of clone A5 (1.0 kb) is highly homologous to a previously cloned cDNA deposited in the GenBank; however, 5' end of A5 differs from this cDNA, and A5 lacks a 66 bp short sequence in the middle. We confirmed existence of A5 transcript by RT-PCR using specific primers, and hypothesize that A5 is a splicing variant of this known sequence. The results of Northern blot hybridization with a probe, which would recognize both A5 and the known sequence, in human cancer cell lines and human breast cancer tissues are shown in Fig. 6. This probe was positive in all the cancer cell lines except a lung cancer cell line. Non-bone metastatic breast cancer cell lines (MDA-MB-361 and MCF7) are also positive. Expression in human breast cancer tissues is higher than in normal control tissues in at least in two of the four cases, although the RNA samples in this particular commercial blot show some degradation. We are currently raising rabbit antibodies against a putative peptide of A5 and that of the known cDNA.

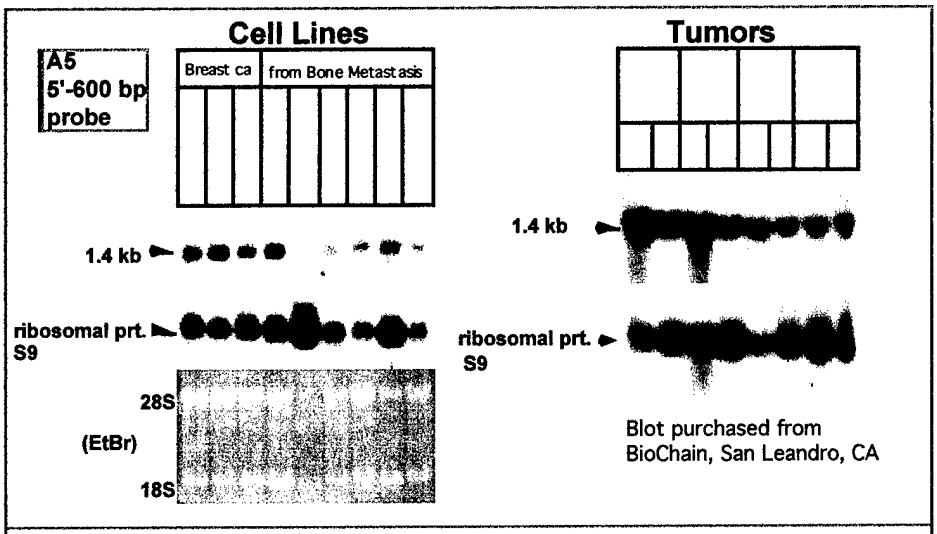

Fig.6 Northern blots with 600 bp A5 probe. Cell lines are as same as the blots with A3 probe. Northern blot shown in the right was a commercial filter containing ten μg of total RNA from human breast cancer tissue and each normal control tissue.

In vivo* experiment to test the ability of CD59-expressing or A3-expressing cells to home to bone marrow *in vivo

We originally planed to visualize the homing of human CD59-expressing cells or A3-expressing cells by immunohistochemistry, since human CD59 and mouse CD59 are quite different (40 % homology in their DNA sequences, 11 % in their amino acid sequences), and endogenous mouse CD59 is unlikely to cross react to antibodies against human CD59. We tested four commercially available antibodies against human CD59, goat polyclonal to N-terminal peptide peptide (Santa Cruz Bio, Santa Cruz, CA), goat polyclonal to C-terminal peptide (Santa Cruz Bio, Santa Cruz, CA), mouse monoclonal clone MEM-43 (Sanbio, Amuden, Netherlands), and mouse monoclonal clone 193-27 (NeoMakers, Fremont, CA). We found that none of these antibodies was suitable for immunohistochemistry on formalin fixed mouse tissue. Therefore we decided to use human placental alkaline phosphatase (PLAP) as a reporter protein to identify injected cells.

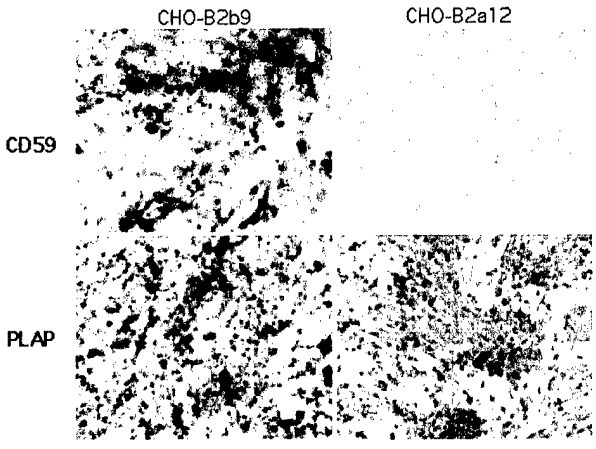

Fig. 8: Alkaline phosphatase staining of the CD59+/PLAP+ clone CHO-B2b9 and the CD59-/PLAP+ control clone CHO-B2a12.

We have an affinity purified rabbit anti-PLAP antibody that works for immunohistochemistry on mouse tissue (Narisawa et al., 1993). Firstly, a transformant clone of Chinese Hamster Ovary (CHO) cells expressing human PLAP (Hummer et al., 1991) was transfected with CD59/pCMV-Script vector and G418 resistant clones were isolated. It was necessary to perform single cloning by limiting dilution, because all the 12 isolated G418+ clones tested contained high number of negative cells. We have selected two CHO cell clones (B2b9 and B2e3) as CD59+/PLAP+ cells and one clone (B2a12) as control cells (CD59-/PLAP +) (Fig. 8).

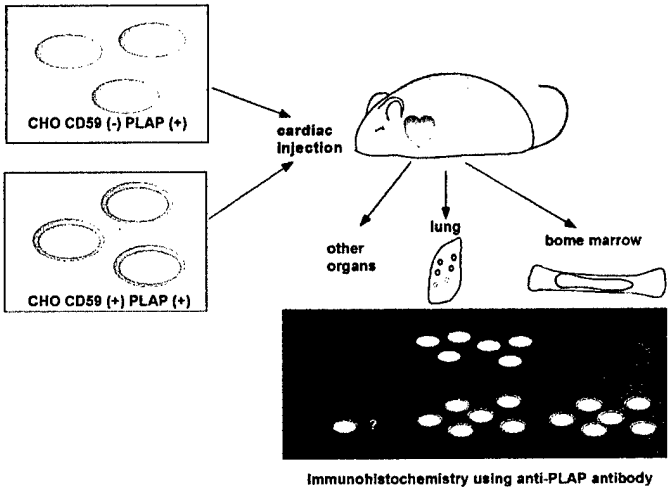

Fig. 9 describes the strategy to be followed to evaluate the specific homing of the transfectants to the bone marrow. The cells will be injected into left cardiac ventricle of Balb/c mouse, and after 1 hour of incubation, the injected mice will be analyzed. We are currently comparing a method with perfusion and exsanguination prior to sampling of the organs.

Fig. 9: Strategy to evaluate specific homing of transfected cells.

A novel *in vivo* expression cloning experiment

In our initial proposal we planed to isolate peptide motifs that home to bone marrow by *in vivo* organ targeting strategy. However, we found that small phage particles (1-2 μm length in filamentous phage) were non-specifically captured by bone marrow cells due to phagocytic activity (most likely, by stromal cells, macrophages and/or endothelial cells). We have now rethought our *in vivo* targeting strategy to bypass those problems. We have used the mammalian expression COS-1 cell cloning system described above and applied it to develop a new *in vivo* expression cloning system as shown in Fig. 10. In this approach plasmids in COS-1 cells will be

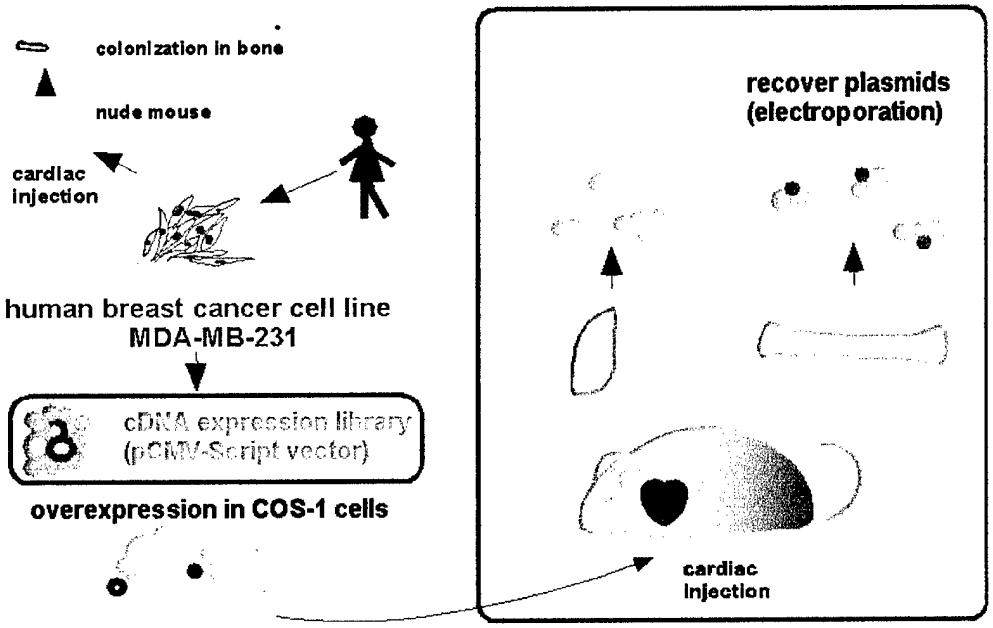

Fig. 10: New *in vivo* targeting using COS-1 cells transfected with MDA-MB-231 cDNA expression library.

recovered from the bone marrow tissue of injected mice. We have obtained a total 20 plasmid clones (3 are the same clones) as shown in below (TABLE). We have chosen 15 clones (bold letters in the table) for individual analysis. COS-1 cells expressing each clone will be injected into mice together with COS-1 cells transfected with the pSVT7 plasmid, which carries an ampicillin resistance gene. The ratio of kanamycin resistant bacteria colonies, which contain the testing cDNA clones in pCMV-Script to the number of ampicillin resistant bacteria colonies, which contain pSVT7, will be compared between the DNA samples recovered from bone marrow and lung. Lung tissue traps COS-1 cells randomly and we use the lung sample as control. Any clone that gives higher ratio of kanamycin resistant colonies in bone marrow than lung, will be considered a candidate clone. We will make stable tansformants of such clones and test bone homing as described above..

TABLE: List of new clones obtained by use of the modified *in vivo* targeting strategy.

clone	bac	size	Blast nr	Blast est	known prt?	predicted.prt	source	chr
26.1	XL10	2.7	AC008114 (BAC)	BE889643	no	?	leiomyosarcoma	12
26.3	XL10	1	AK001750			FLJ10888	terato ca	4
26.4	XL10		AF347002		mitochondrial DNA			mitc
11.1	XL-B	1.5	XM_002665.6	BF593640	Cys-rich protein		moter neuron, glioblastoma	4
11.2	XL-B				ribosomal protein S30		hypothalamus	11
11.4	XL10	1.8	XM_028947.1	BI463917		FLJ10468	testis	1
11.6	XL10	0.85*	XM_004593.1	BI115910	sorcin (ca binding prt)		lung small cell ca, MGC3	7
11.7	XL10		BC005002	BI834883	mitochondria ribosomal		lymphoma,pancr.spleen	5
11.8	XL10	0.35	AC092849.4	AI524014	no	ORF 155 aa	B-cell leukemia	7
11.9	XL10	1.3	BC014391	BI911460	no	ORF 219 aa	leukocyte, renal ad.ca	2
11.10	XL10	2.0**	XM_048403.1	BI011099	filamin A, actin binding prt		normal lung,	X
11.11	XL10	1.6	BC001357		no	SP260, FLJ10243	melanoma	
11.12	XL10	0.65	XM_001468		calpactin I(p11)ca-binding			1
11.13	XL10	0.28***	?		unknown			1
11.14	XL10	0.65	XM_001468		calpactin I(p11)ca-binding			
11.15	XL10	1.5	XM_002665.6		Cys-rich protein		moter neuron, glioblastoma	4
11.16	XL10	1.7	BC001590		man.6 P receptor binding prt		colon adeno ca	19
11.17	XL10		AB009010		ubiquitin C			
11.18	XL10	0.28***	?		unknown			12
11.19	XL10	1.4	NM003299.1		tumor rejection antigen(gp96)		terato ca LASTD	
11.20	XL10	0.6	XM_03470.1		DAD-1		Raji	

* 0.4+0.45
**1.4+0.6
***0.18+0.1

7. KEY RESEARCH ACCOMPLISHMENTS

- Characterization of bone marrow stromal and endothelial cell lines
- Development of two binding assays to be used in expression cloning by an *in vitro* panning procedure
- Cloning and initial characterization of two novel cDNAs mediating the binding of a mestastatic breast cancer cell line to an immortalized bone marrow stromal cell line.
- Development of an *in vivo* screening procedure for organ-specific targeting
- Development of a novel *in vivo* targeting strategy to obviate the phagocytosis problem associated with the use of random peptide library *in vivo* targeting based on phage technology.

8. REPORTABLE OUTCOMES

A manuscript describing these novel panning and *in vivo* targeting strategies is in preparation.

9. CONCLUSIONS

We have requested and received approval to extend the duration of our approved grant until July 2002, without additional funds. This extra time will allow us to complete the studies and publish our conclusions. During this final year of support, we will complete the characterization of clone A3 and A5, which appear to be novel molecules. We will complete the evaluation of the specific targeting properties of these two molecules using the *in vivo* strategy outlined above. We will also initiate a thorough analysis of the additional 15 sequences that were identified by the new *in vivo* targeting strategy.

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